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DETERMINATION OF BINDING CONSTANTS OF WATER SOLUBLE CHEMICALS FOR BIOLOGICALLY BASED KINETIC MODELS

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FOR THE DIRECTOR

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The interaction of exogenous chemicals (xenobiotics) with endogenous biomolecules can modulate the kinetics of that chemical in biological systems. The purpose of this report is to provide a fundamental understanding of the underlying mathematical and experimental principles involved in describing these interactions. The theoretical basis of the interaction of xenobiotics with binding sites located on endogenous molecules are discussed in the context of the following scenarios: (i) interaction between a single chemical and a single class of binding sites, (ii) interaction between a single chemical and multiple classes of binding sites, (iii) interactions between multiple chemicals and a single class of binding sites. Each of these situations is analyzed in the context of the reaction mechanisms involved and the mathematical description of those reactions. Experimental approaches to evaluating the basic mechanisms of binding and estimation of binding parameters using titration experiments and inhibition experiments are described. Practical instructions are provided for the conduct and interpretation of these experiments. The relationship between theoretical considerations and practical experimental consequences are highlighted.

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PREFACE

This report is one of a series of reports describing the research efforts of the Predictive Toxicology program at AFRL/HEST. This document serves as an interim report of the Predictive Kinetics task. The objectives of this task is to develop innovative approaches to predicting the kinetics of chemicals of interest to the Air Force in mammalian systems. The research effort focuses on the use of in vitro experimental techniques to evaluate kinetic parameters for use in biologically based kinetic models to simulate in vivo chemical kinetics. An important process in controlling the kinetics of chemicals in mammalian systems is the binding of the chemical to endogenous molecules. The purpose of the research described in this document was to provide guidance for the evaluation of binding parameters using in vitro methods. The research described in this report began in November 1997 and was completed in October 1998. This work was financially supported by the Air Force Office of Scientific Research (2312A202). Technical support was provided by ManTech Geo-Centers Joint Venture F41624-96-C-9010. Maj Steven Channel served as Contract Technical Monitor for AFRL/HEST. No animals were used in the studies described in this document.

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1.0 INTRODUCTION

The interaction of an exogenous chemical with an endogenous biomolecule[1] can be a potential modulator of the kinetics of that chemical in the biological system of interest. An understanding of the underlying mathematical principles involved in describing these interactions is essential not only in biologically based kinetic (BBK) modeling of the toxicokinetics of the chemical, but also in the design and interpretation of the results of chemical binding assays (Fang and Lindstrom, 1980; Taira and Terada, 1985).

In this report, binding refers to the direct molecular interaction between the chemical and an biomolecule. Binding can be specific or non-specific. Specific binding implies that the three-dimensional atomic architecture at the molecular binding site on the biomolecule imparts specificity to the interaction between the chemical and the biomolecule. In this case, the affinity (strength) of the binding is usually very high for the chemical and structurally related analogues, and low for structurally unrelated chemicals. Non-specific binding is usually of lower affinity and does not have strong topological selectivity. Finally, in some cases where hydrophobic regions of significant size exist in an endogenous macromolecule, a phase partitioning can occur, i.e., the chemical can "dissolve" in the "lipid" phase of the macromolecule. This type of interaction implies that the chemical has strong hydrophobic tendencies and, therefore, would not be particularly water soluble. Since this report deals with water soluble chemicals this type of apparent binding will not be considered further.

This report discusses the theoretical aspects of the interactions of exogenous chemicals with binding sites located on endogenous molecules. The following scenarios are discussed:

(i) interaction between a single chemical and a single class of binding sites

(ii) interaction between a single chemical and multiple classes of binding sites

(iii) interactions between multiple chemicals and a single class of binding sites

[1] In the context of this discussion of the binding of exogenous chemicals to endogenous molecules, the focus is on the behavior of the exogenous chemical. Therefore, the term *chemical* will refer in all cases to the exogenous chemical. The term *biomolecule* will be used to identify any endogenous chemical entity (from low molecular weight molecules to macromolecules) that can reversibly bind the chemical under consideration. The term *binding site* will refer to the specific portion of the biomolecule that interacts with the chemical.

Each of these situations will be analyzed in the context of the reaction mechanisms involved and the mathematical description of those reactions. The relationship between theoretical considerations and practical experimental consequences will be highlighted.

The experimental approach to evaluating the basic mechanisms of binding and estimation of binding parameters consists of three types of experiments: (1) titration experiments, (2) inhibition experiments, and (3) kinetic experiments. In titration experiments, the concentration of biomolecule of interest is held constant and the amount of chemical is varied. From this type of experiment, the binding capacity, as measured by the concentration of binding sites, and the affinity of the binding site for the chemical can be estimated. In inhibition experiments, the titration experiment is conducted in the presence of varying concentrations of the inhibitor and the effect of competition on the binding of the chemical of interest is evaluated. Finally, in the kinetic experiments, the concentrations of biomolecule and chemical are held constant and the interaction is investigated over time. From kinetic experiments, the forward and reverse reaction rate constants can be estimated. This report concentrates on the first two types of experiments and practical instructions will be provided for the conduct and interpretation of these experiments. Kinetic experiments are only briefly mentioned.

2.0 THEORY

2.1 SPECIFIC BINDING: SINGLE CHEMICAL - SINGLE CLASS OF BINDING SITES

The analysis of the reaction of a chemical with a single class of binding sites is based on the following assumptions:

- Binding obeys the law of mass action
- Binding is reversible.
- All binding sites are equivalent
- There is no interaction between binding sites, i.e., binding of the chemical to one binding site does not alter the affinity of any other binding site for the chemical.
- Both the chemical and the biomolecule are homogeneously distributed in the reaction volume, i.e., all molecules of the chemical and the biomolecule of interest have equal probability of interacting.
- Both chemical and binding sites are univalent, i.e., one molecule of chemical will react with one binding site.
- All binding sites are either free or are complexed with the chemical. There are no intermediate states of partial binding.
- Neither the chemical nor the binding site is permanently altered by the binding reaction.
- When radio-labeled chemical is used, the radio-labeled and unlabeled chemical have the same physical-chemical properties, Note, isotope effects on reaction kinetics may be important for low molecular weight chemicals.

If any of these assumptions are not satisfied, then the analysis below will not provide an adequate description of the binding reactions.

Based on these assumptions, chemical binding is described by the law of mass action (Chapter 3: Gutfreund, 1995):

$$T + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} TL \qquad (1)$$

where T represents the chemical (toxicant) of interest, L represents the binding site, TL represents the chemical-binding site complex, k_{+1} $\{\mu M^{-1} * min^{-1}\}$ is the association rate constant and k_{-1} $\{min^{-1}\}$ is the dissociation rate constant. Binding occurs when the chemical and the binding site approach each other due to random diffusion. The chemical-binding site complex forms provided the collision has the correct orientation and sufficient energy to overcome any activation energy barriers. The forward (association) reaction rate is given by:

$$R_{FORWARD} = k_{+1} * F * [L] = k_{+1} * F * (B_{MAX} - B) \qquad (2)$$

where $R_{FORWARD}$ $\{\mu moles\ bound * min^{-1} * L^{-1}\}$ is the forward reaction rate, F $\{\mu M\}$ is the concentration of the available free chemical, [L] $\{\mu M\}$ is the concentration of unoccupied binding sites, which is equal to the concentration of the total number of binding sites, B_{MAX}, less those sites that are occupied, as represented by the concentration of bound chemical, B $\{\mu M\}$. Once binding has occurred, the chemical and the binding site remain associated for an period of time, the duration of which is stochastic in nature and influenced by the strength of the binding interaction between the chemical and the binding site.

The rate of the reverse (dissociation) reaction is:

$$R_{REVERSE} = k_{-1} * [TL] = k_{-1} * B \qquad (3)$$

where $R_{REVERSE}$ $\{\mu moles\ dissociated * min^{-1} * L^{-1}\}$ is the rate of dissociation, [TL] $\{\mu M\}$ is the concentration of the chemical-binding site complex, which is equal to the bound concentration of the chemical, B.

Equilibrium is reached when the rate at which chemical-binding site complexes are formed equals the rate at which the complexes dissociate, i.e., when

$$R_{FORWARD} = R_{REVERSE} \qquad (4)$$

Substituting for the forward (Equation 2) and reverse (Equation 3) rates, we have

$$k_+ * F * (B_{MAX} - B) = k_- * B \quad (5)$$

and solving for the bound concentration, B, gives

$$B = \frac{B_{MAX} * F}{F + K_D} \quad (6a)$$

where

$$K_D = \frac{k_{-1}}{k_{+1}} \quad (6b)$$

K_D {μM} is the dissociation equilibrium constant that relates the free and bound concentrations at equilibrium. A small K_D means that the binding site has a high affinity for the chemical and vice versa, a large K_D means that the binding site has a low affinity for the chemical. It should be emphasized that k_- and K_D are not the same and they are not even expressed in the same units. The K_D is the dissociation equilibrium constant, whereas k_- is the dissociation rate constant. Note, the equilibrium relationship is sometimes discussed in terms of the association reaction. In this case, the equilibrium is defined by the association equilibrium constant, which is the reciprocal of the dissociation equilibrium constant.

The standard binding curve is illustrated in Figure 1. Figure 1A demonstrates the effect of varying the binding affinity, as described by the dissociation equilibrium constant, K_D, while keeping the binding capacity, B_{MAX} = 100 μM, constant. As the dissociation equilibrium constant, K_D, increases, the affinity decreases. Thus, as the K_D increases, higher free concentrations are required to saturate (i.e., fully occupy) the available binding sites. Figure 1B demonstrates the effect of varying the capacity, B_{MAX}, while maintaining a constant affinity, K_D = 10 μM. As the capacity increases, the amount bound at a given free concentration increases. Thus, both K_D and B_{MAX} have an impact of the behavior of chemical binding. Note, when the free concentration of the chemical is equal to the K_D (= 10 μM), the binding sites are half saturated, i.e., one half of the total binding sites are occupied.

The dissociation constant determines the distribution of the chemical between the free and bound forms at equilibrium and is a thermodynamic property of the chemical - binding molecule system. K_D does not determine the time required to attain equilibrium which is controlled by the individual rate constants. The individual microscopic rate constants can become important if they are kinetically rate limiting, i.e., result in binding reactions that are slow relative to rates for other processes of interest. Thus, a slow dissociation rate constant (again, not to be confused with the equilibrium dissociation constant) means that a bound chemical may not come off the binding site rapidly enough during transit through a tissue to be involved in various kinetic processes, such as

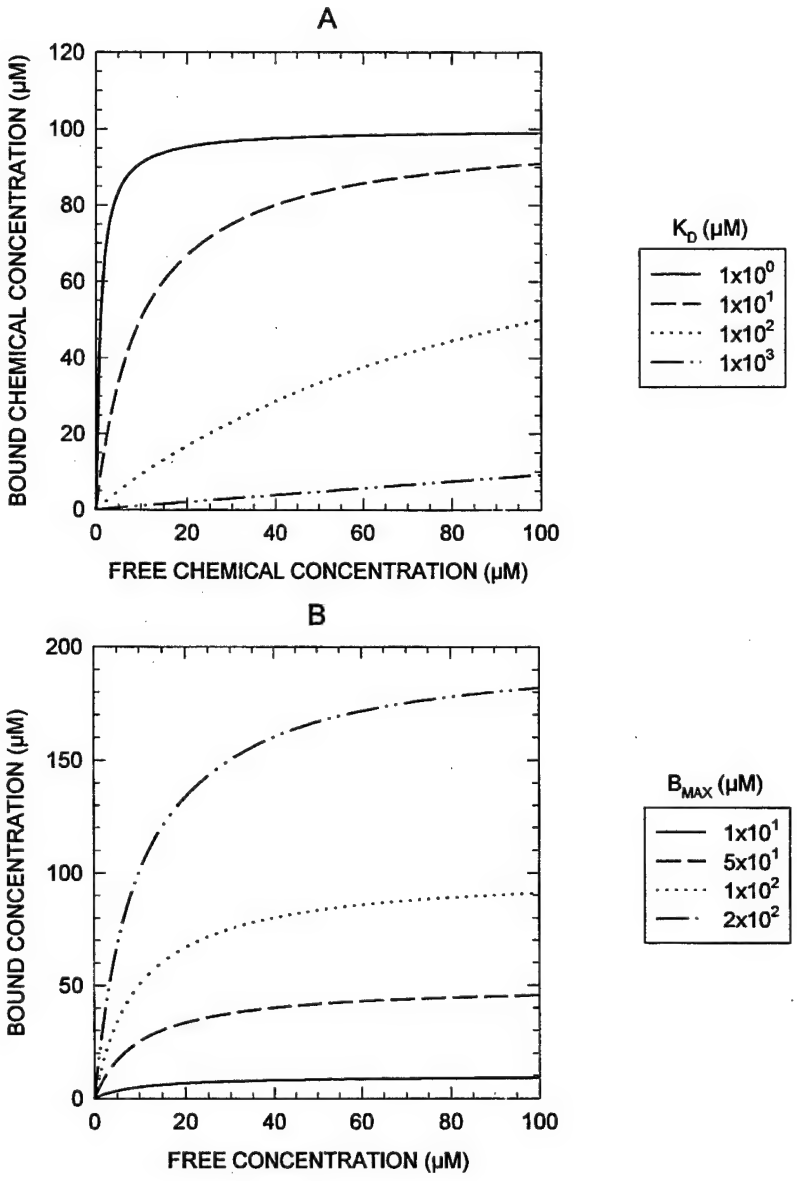

Figure 1: Standard binding curves - one class of binding sites. (A) The effect of varying binding affinity, K_D. The binding capacity, B_{MAX}, was held constant (B_{MAX} = $1x10^2$ μM) and the K_D was varied from 1 to $1x10^3$ μM. (B) The effect of varying B_{MAX}. The binding affinity, K_D, was held constant (K_D = $1x10^1$ μM) and the B_{MAX} varied from $1x10^1$ to $2x10^2$ μM.

transport or metabolism. If the dissociation rate constant is extremely small, the binding appears to be irreversible from a practical point of view.

In BBK models, it is often assumed that the rate of various kinetic processes, such as membrane transport and metabolism, depends on the free chemical concentration. It is possible to estimate the theoretical free concentration in solution given the total chemical concentration and the binding parameters - B_{MAX} and K_D. The total concentration of the chemical is equal to the sum of the free and bound species. Thus

$$Z = F + B = F + \frac{B_{MAX} * F}{F + K_D} \tag{7}$$

where Z {μM) is the total concentration of the chemical. This equation can be solved for the free concentration giving a quadratic equation in F which can be solved:

$$F = \frac{1}{2}\left(\sqrt{(K_D + B_{MAX} - Z)^2 + 4 * K_D * Z} - (K_D + B_{MAX} - Z)\right) \tag{8}$$

For real, positive values of K_D, B_{MAX} and Z, i.e., physiologically meaningful values, the solution for the free concentration will be real and positive. This equation can be used to compute the free concentration in the presence of a single class of binding molecule. It can be used in BBK models to determine the free concentration of the chemical needed for the differential equations that describe the chemical kinetics. A plot of the free concentration as a function of the total concentration is given in Figure 2. Figure 2A illustrates the dependency of the relationship on the dissociation equilibrium constant, K_D. As K_D increases, the effect of binding on the free concentration decreases. Figure 2B illustrates the influence of the binding capacity, B_{MAX}. The greater the binding capacity, the less free chemical present for a given total chemical concentration. In all cases, the presence of binding reduces the free chemical concentration.

2.2 SPECIFIC BINDING: SINGLE CHEMICAL - MULTIPLE CLASSES OF BINDING SITES

In many situations, more than one class of binding site is present. This leads to a more complicated description of the binding relationships. For the following discussion, we will assume that there are only two classes of binding sites and that binding at one site has no effect on the binding of the chemical to any other site, either of the same class or the other class.[2] The proposed reactions are:

$$T + L_1 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} TL_1 \tag{9a}$$

[2] The general case of more than two classes of binding sites can be evaluated by analogy to the discussed here.

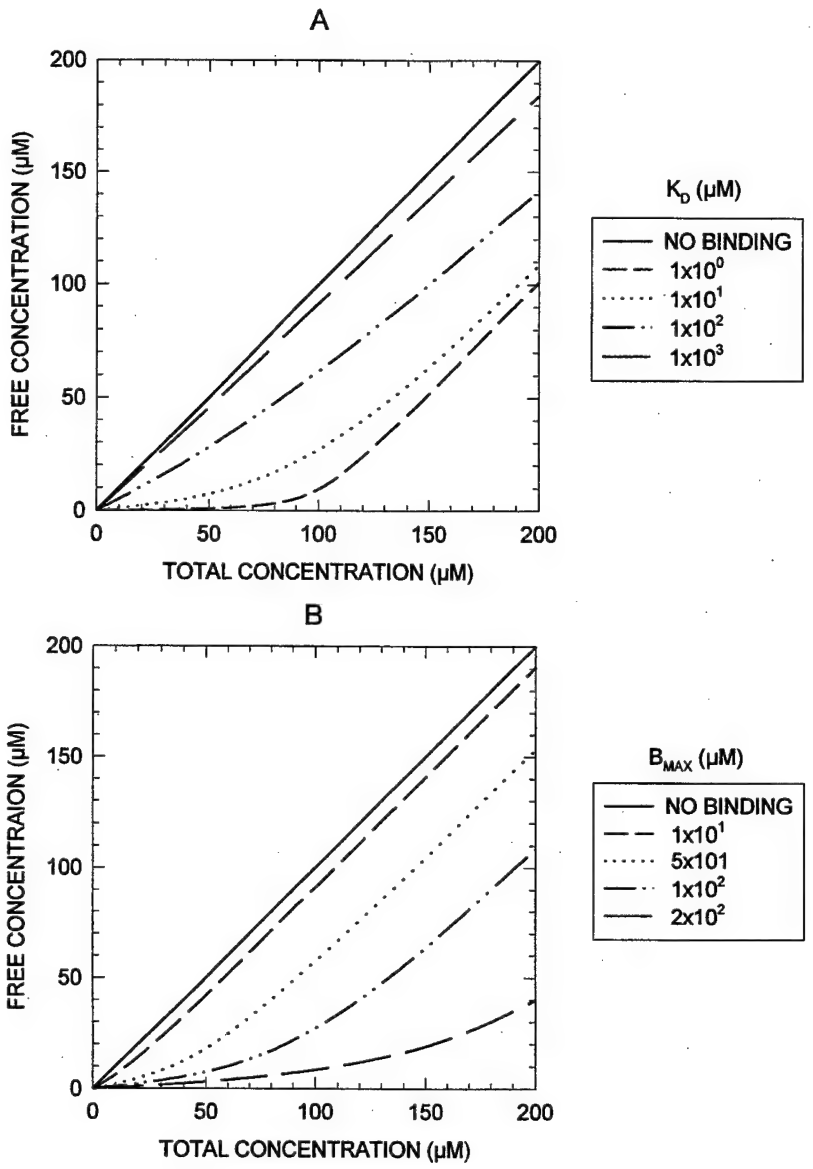

Figure 2: The relationship between free chemical concentration and total chemical concentration - one class of binding sites. (A) The effect of varying binding affinity, K_D. The binding capacity, B_{MAX}, was held constant (B_{MAX} = $1x10^2$ µM) and the K_D was varied from 1 to $1x10^3$ µM. (B) The effect of varying B_{MAX}. The binding affinity, K_D, was held constant (K_D = $1x10^1$ µM) and the B_{MAX} varied from $1x10^1$ to $2x10^2$ µM.

and

$$T + L_2 \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} TL_2 \qquad (9b)$$

where L_1 and L_2 represent the two classes of binding sites. In this case, the law of mass action can be applied to both binding reactions at equilibrium, giving

$$k_{+1} * F * (B_{MAX1} - B_1) = k_{-1} * B_1 \qquad (10a)$$

and

$$k_{+2} * F * (B_{MAX2} - B_2) = k_{-2} * B_2 \qquad (10b)$$

where B_1 and B_2 {μM} are the concentrations of the chemical bound to binding site 1 and binding site 2, respectively, and B_{MAX1} and B_{MAX2} are the total concentrations of binding site 1 and binding site 2, respectively. Since binding to one class of binding sites has no interaction with binding to the other class of binding sites, the two equations are independent and can be solved for the bound concentration as a function of the free concentration:

$$B_1 = \frac{B_{MAX1} * F}{F + K_{D1}} \qquad (11a)$$

and

$$B_2 = \frac{B_{MAX2} * F}{F + K_{D2}} \qquad (11b)$$

Each of these relationships are of the form discussed above for the single binding site situation and give binding curves similar to Figure 1. B_{MAXi} and K_{Di} have the same interpretations.

The total bound concentration is

$$B = B_1 + B_2 = \frac{B_{MAX1} * F}{F + K_{D1}} + \frac{B_{MAX2} * F}{F + K_{D2}} \qquad (12)$$

Experimental binding studies will only measure the total concentration of bound chemical and the free concentration. When the total bound concentration is plotted against the free concentration binding curves such as Figure 3 are obtained. In Figure 3A, two classes of binding molecules are present with equal binding capacity, B_{MAX1} =

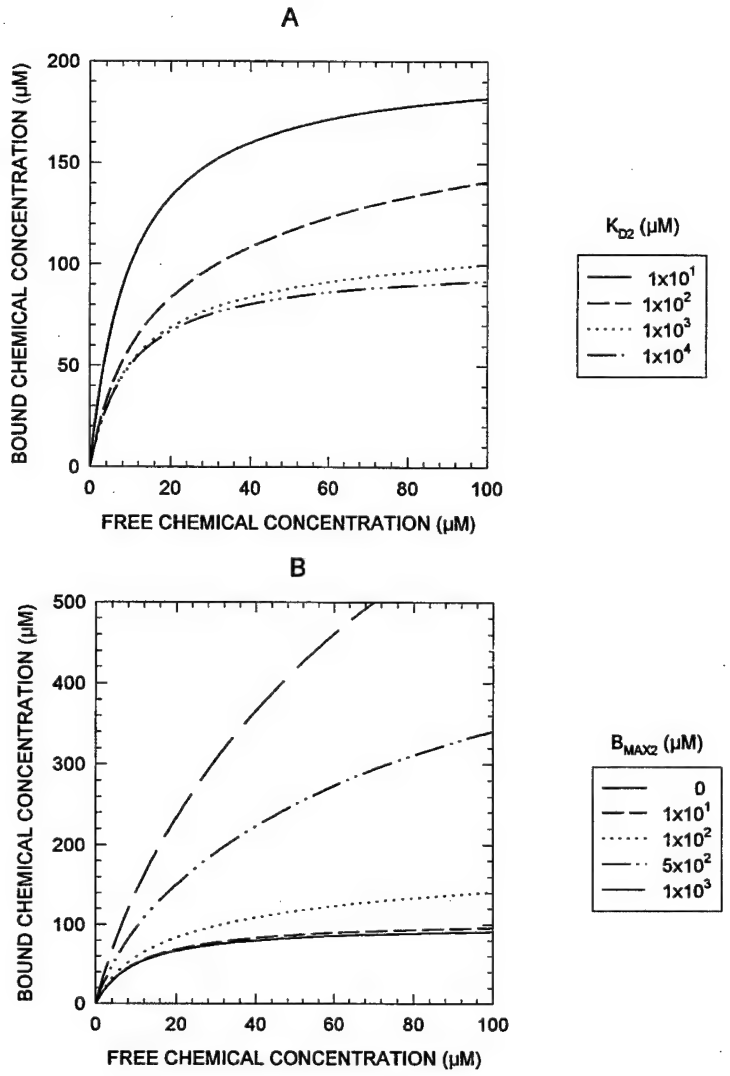

Figure 3: Standard binding curves - two classes of binding sites. (A) The effect of arying binding affinity of the second class of binding sites, K_{D2}. For the first class of inding sites B_{MAX1} = 100 μM and K_{D1} = 10 μM; for the second class of binding sites $_{MAX2}$ = 100 μM and K_{D2} varied from 10 to $1x10^4$ μM. (B) The effect of varying the binding capacity of the second class of binding sites. For the first class of binding sites B_{MAX1} = 100 μM and K_{D1} = 10 μM; for the second class of binding sites K_{D2} = 100 μM and B_{MAX2} varied from 0 to $1x10^3$ μM.

B_{MAX2} = 100 μM, but with different affinities, K_{D1} = 10 μM and K_{D2} varied from 10 to 1000 μM. It is obvious by looking at Figure 3A that it would be difficult to identify the presence of two classes of binding sites by just inspecting the binding curve, particularly if there is experimental error present. As it turns out, there are ways to transform the data that will provide better visual discrimination between one and multiple binding site situations (see Section 3.1.1).

The two binding reactions described above are not totally independent since the binding of chemical molecules to one binding site reduces the number of molecules of the chemical available to bind to the other binding site. This interaction is taken into account by the conservation of mass relationship

$$Z = F + B_1 + B_2 = F + \frac{B_{MAX1} * F}{F + K_{D1}} + \frac{B_{MAX2} * F}{F + K_{D2}} \qquad (13)$$

Solving this equation for the free concentration of the chemical as a function of the total concentration gives a cubic equation which can be solved numerically by the Newton-Raphson technique. The graphical form of the relationship between free chemical concentration as a function of total chemical concentration is similar to that for the single class of binding sites (Figure 2).

2.3 SPECIFIC BINDING: MULTIPLE CHEMICALS - SINGLE CLASS OF BINDING SITES - INHIBITOR REACTIONS

The objective of studies designed to investigate the influence of structurally related chemicals on the interaction of a particular chemical with a biomolecule is to explore the nature of the binding site, e.g., to determine whether the binding is specific or nonspecific. Such studies provide useful information about the structural determinants that confer specificity to the chemical-binding site interaction. It is also important from a toxicological point of view to identify other chemicals that will interact with a given binding site in order to understand the spectrum of potential chemical interactions.

Consider the general case of two chemicals that can interact with the same binding site. One chemical is the chemical of interest and the other is referred to as the binding inhibitor. The total concentration of the binding site is B_{MAX}. Both the chemical and the inhibitor will react with the binding site by mass action. Thus, forward and reverse reactions for each chemical can be written similar to Equations 2 and 3 where a subscript must be introduced to identify the inhibitor. In Equation 2 the concentration of free binding sites is eliminated from the equation by using the mass balance relation, i.e., $[L] = B_{MAX} - B$ where B was the concentration of bound chemical. In the presence of two chemicals that are competing for binding to the same binding site, this relationship must be modified to account for all the binding sites that are occupied, either by the chemical or the inhibitor. Thus, $[L] = B_{MAX} - B - B_I$, where B is the bound concentration of the chemical and B_I is the bound concentration of the inhibitor. This relationship can

be used to eliminate the concentration of unoccupied binding sites in both equations for the forward reactions. At equilibrium there are two algebraic equations that must be solved simultaneously

$$k_{+1}*F*(B_{MAX} - B - B_I) = k_{-1}*B \quad (14a)$$

and

$$k_{I+1}*F_I*(B_{MAX} - B - B_I) = k_{I-1}*B_I \quad (14b)$$

The second equation can be solved for B_I and then used to eliminate B_I from the first equation. After algebraic rearrangement of the first equation it can be shown that

$$B = \frac{B_{MAX}*F}{F + K_D*\left(1 + \frac{F_I}{K_I}\right)} \quad (15a)$$

where

$$K_I = \frac{k_{I-1}}{k_{I+1}} \quad (15b)$$

This is the traditional competitive inhibition relationship. If the concentration of the inhibitor is zero, then the equation reduces to the simple relationship for a single chemical interacting with a single binding site (Equation 6a). The effect of the inhibitor is to increase K_D by the factor $(1 + F_I/K_I)$ without affecting the maximum binding capacity, B_{MAX}. The quantity $K_D*(1+F_I/K_I)$ can be considered the apparent K_D in the presence of the inhibitor. Remember, an increase in the apparent K_D implies a reduction in the apparent affinity of the chemical for the binding site. Thus, a competitive binding inhibitor has the effect of reducing the affinity of the binding site for the chemical of interest.

The effect of a competitive binding inhibitor on the binding of the chemical of interest to the binding site is illustrated in Figure 4. Figure 4A demonstrates the effect of the affinity of the inhibitor, K_I, on chemical binding. As the strength of the inhibitor-binding site interaction increases, K_I decreases and the amount of chemical bound at a given free chemical concentration decreases. When this effect is translated into the effect of the inhibitor on the concentration of free chemical at a given total chemical concentration (Figure 4B), it is clear that as the affinity of the inhibitor increases, the concentration of free chemical increases, to the point where there appears to be no binding at all. In practical terms, when there is a competitive binding inhibitor present, there is more free chemical present to support kinetic processes such as membrane transport, metabolism, etc. Furthermore, there is more free chemical to react with molecular targets for toxicity.

In BBK models, the calculation of the free chemical concentration in the presence

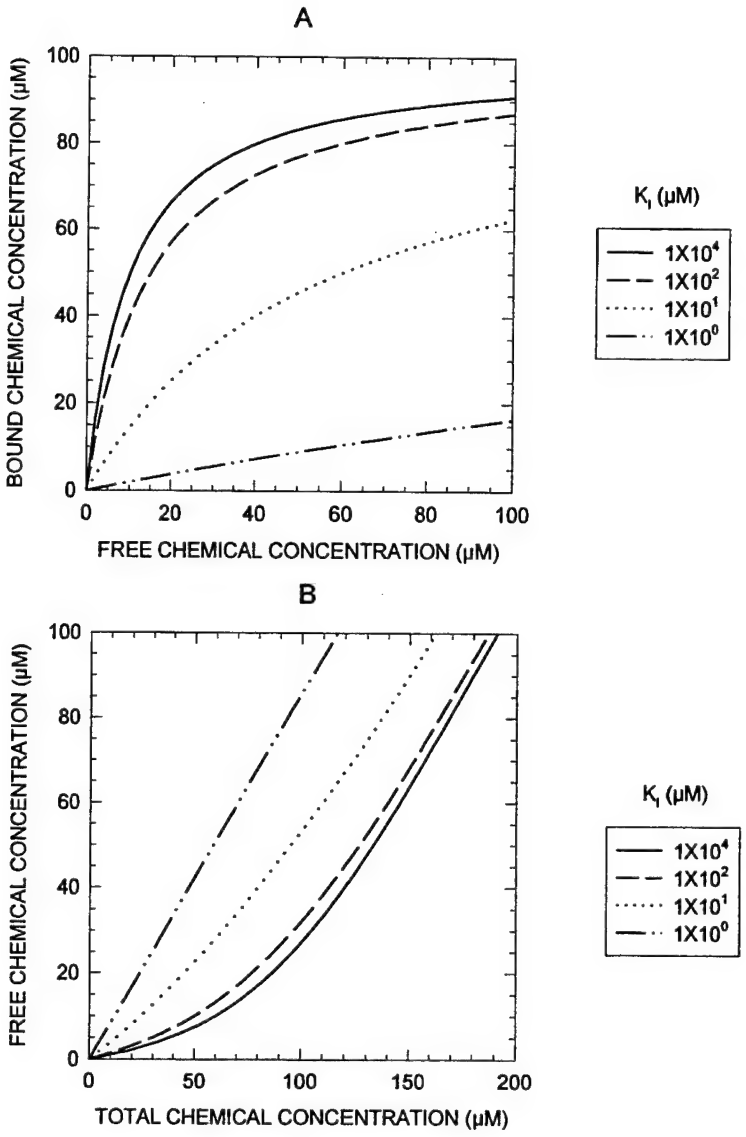

Figure 4: The effect of a competitive binding inhibitor on chemical binding. (A) Effect of inhibitor on the standard binding curve. The binding parameters for the chemical of interest are B_{MAX} = 100 μM and K_D = 10 μM; the binding inhibitor has a B_{MAXI} = 100 μM and K_{DI} varied from 1 to $1x10^4$ μM. (B) Effect of inhibitor on the relationship between free and total chemical concentration. Parameters are the same as in (A).

of an endogenous inhibitor results in a slight modification of Equation 8. If the concentration of exogenous chemical present in a compartment is relatively low, so that the equilibrium relationship between the free and bound species of the endogenous inhibitor is relatively undisturbed, then the apparent K_D can be used in Equation 8 to calculate the free concentration of the chemical of interest. If however, the concentration of the exogenous chemical is sufficient high to alter the equilibrium status of the endogenous inhibitor, i.e. F_I is significantly altered from the free concentration in the absence of the chemical of interest, then it will be necessary to solve the simultaneous set of two binding equations:

$$Z = F + \frac{B_{MAX} * F}{F + K_D * \left(1 + \frac{F_I}{K_I}\right)} \tag{16a}$$

and

$$Z_I = F_I + \frac{B_{MAX} * F_I}{F_I + K_I * \left(1 + \frac{F}{K_D}\right)} \tag{16b}$$

Given the total concentration of both the chemical of interest, Z, and that of the inhibitor, Z_I, and the binding parameters for both chemicals, the two non-linear equations above must be solved simultaneously for the free concentrations, F and F_I, that satisfy the relationships.

2.4 NON-SPECIFIC BINDING REACTIONS

In addition to specific binding of chemicals to defined binding sites present on endogenous molecules, chemicals can bind to many biological molecules at sites that have little structural specificity. In general, non-specific binding is weaker than specific binding and structurally unrelated chemicals can compete for binding to non-specific binding sites. Non-specific binding can be a confounding factor when trying to investigate the nature of specific binding sites. If there is significant non-specific binding, then the behavior of the specific binding sites may be masked. Non-specific binding is usually linear with respect to the free concentration of the chemical within the physiological range. Thus, non-specific binding, denoted B*, can be described by a linear relation:

$$B^* = \alpha * F \tag{17}$$

where α {dimensionless} is the linear binding constant. In principle, the concept of non-specific binding is somewhat arbitrary. Even non-specific binding has some characteristics that are dependent on molecular structure of the chemical, e.g., charge distribution, dipole moment, hydrogen binding sites, etc. Furthermore, the capacity for nonspecific binding is not infinite. Since the binding tends to be weaker for the

nonspecific sites, either the limit of solubility of the chemical is reached before saturation of binding capacity becomes important or alterations of the binding molecule occur (e.g., denaturation of a protein molecule). Thus, within the range of physiologically relevant concentrations, the linear binding relationship is usually adequate.

From the point of view of kinetic modeling, it does not matter whether binding is specific or non-specific unless chemical interactions are important. Binding of either form reduces the free concentration of the chemical of interest and will impact on kinetic processes. Thus, all forms of binding must be taken into consideration when developing a BBK model.

3.0 EXPERIMENTAL METHODS

3.1 TITRATION (EQUILIBRIUM BINDING) EXPERIMENTS

3.1.1 Experimental Design of Titration Experiments

In a typical binding experiment, one mixes the chemical and the endogenous molecule of interest in a reaction vessel and allows time for the mixture to reach equilibrium. Next, various physical-chemical techniques are used to separate the components of the mixture so that any two of the three quantities, free chemical, bound chemical or total chemical concentration, can be measured. Given any two concentrations the third can be computed. The basic principles of this method requires that two experimental conditions exist:

(i) Either the bound or free species of the chemical can be separated perfectly without perturbing the equilibrium, and
(ii) The concentrations of the two quantities measured (usually the total and the free concentrations) can be determined accurately so that errors in the third quantity (usually the bound concentration determined as the difference between the total and free concentrations) are not excessive.

Assuming these conditions are met, the experiment is conducted by carrying out the equilibrium reactions for a series of total concentrations for the chemical and a fixed concentration of the binding sites. The set of data obtained from the series of equilibrium reactions are used to evaluate the binding interaction.

3.1.2 Experimental Methods

There are two main experimental approaches to evaluation of the interactions of chemicals with endogenous molecules: dialysis and ultrafiltration. Brief descriptions of these two methods are provided.

Dialysis

Dialysis experiments are based on the principle that the endogenous binding molecule of interest can be maintained in a confined space by a semi-permeable membrane. The membrane must allow diffusion of the chemical of interest but be impermeable to the binding molecule. If a membrane that satisfies this condition exists, then the binding study is conducted by using the membrane to divide a temperature controlled reaction vessel into two chambers. Into one chamber is placed a solution of the endogenous molecule, either purified or in a complex mixture. The chemical of interest, dissolved in a buffered solution, is placed into the other chamber and the reaction allowed to proceed to equilibrium. In this system, there are two processes that determine the time it takes to reach equilibrium, diffusion of the chemical into the chamber containing the binding molecule and the binding reaction itself. Usually, diffusion is the rate limiting factor. Once equilibrium is established (the time to equilibrium must be confirmed in preliminary experiments), samples of the solution in the two chambers are collected and the total concentration of the chemical in each chamber is determined by an analytical technique that will measure the total amount of chemical, even in the presence of the binding molecule (or any other bio-components, such as tissue homogenates). For practical purposes, the use of a radiolabeled chemical will allow for the measurement of total chemical concentration.

The measured concentration of the chemical in the chamber without the binding molecule is assumed to be equal to the free concentration of the chemical in equilibrium with the binding sites in the other chamber. Thus, the bound concentration is equal to the total concentration in the chamber with the binding ligand minus the concentration in the chamber without the binding molecule. Note, the concentration of the binding molecule should also be determined to assure that the molecule has remained in solution at the nominal concentration. The data obtained in these studies can be used to generate the standard binding curves and analysis proceeds as described below.

Ultrafiltration

Ultrafiltration techniques also employ a semi-permeable membrane to separate the free chemical from the bound chemical, but in a manner different from that used in the dialysis experiments. In this technique, the chemical is mixed with a solution containing the binding molecule in a reaction vessel and the binding reaction is allowed to reach equilibrium. This reaction should proceed much more rapidly than in the dialysis experiments since diffusion through the membrane is eliminated. Once the reaction has reached equilibrium, the mixture of chemical and binding molecule is placed in an apparatus that can filter the mixture through a semi-permeable membrane. The driving force for ultrafiltration is attained either by high pressure in the head space above the solution, usually using nitrogen gas, or by centrifugation. A small aliquot of the ultrafiltrate is collected and both the starting solution and the ultrafiltrate are analyzed for the total concentration of the chemical of interest (and the binding molecule if possible). Here, the concentration of the chemical in the ultrafiltrate is assumed to be equal to the free concentration of the chemical in equilibrium with the binding sites. The bound chemical concentration is calculated as the difference between the concentration

in the unfiltered mixture and that in the ultrafiltrate. The data are used to construct the binding curves.

3.1.3 Data Analysis for Titration Experiments

To focus the discussion, it is assumed that the experimental measurements made are of the total concentration, Z {μM}, and the free concentration, F {μM), of the chemical in each reaction. This would be the usual case for either dialysis or ultrafiltration studies (see descriptions of these methodologies above). Then the bound concentration is obtained by difference, B = Z - F (μM). Note, the error in the measurement of B will be a combination of the errors in Z and F. The first analysis of the experimental data is always a plot of the bound concentration versus the free concentration. This plot should exhibit a saturation profile as in Figure 1. If the plot appears to be linear and the concentration range used in the study covers the physiologically relevant range, the usual interpretation would be that there is some form of weak (non-specific) binding reaction between the chemical and the binding sites. This situation can be empirically described by a linear binding relationship and characterized by a single linear binding parameter. Another possibility is that there is some curvature in the plot, suggesting saturation, but not clearly reaching an asymptotic value. In this situation, it may be necessary to conduct additional binding studies at higher concentrations of the test chemical to elucidate the exact nature of the binding interaction. There is a limit to the range of concentrations of chemical used in these studies determined by either the solubility of the chemical or the possibility of chemically denaturing the endogenous molecule. Either eventuality ultimately determines the maximum chemical concentration that can be used in the study without introducing artifacts.

Assuming that the B versus F graph give a reasonable approximation to a saturation profile, the next step is to determine the number of different classes of binding sites. This is best accomplished by Scatchard analysis. The theoretical basis of the Scatchard plot is derived from Equation 6a. Multiplying both sides of Equation 6a by $(F + K_D)$, dividing by F and solving for B/F gives:

$$\frac{B}{F} = \frac{B_{MAX}}{K_D} - \left(\frac{1}{K_D}\right) * B \qquad (18)$$

Thus, a Scatchard plot of B/F versus B should give a straight line with y-intercept of B_{MAX}/K_D and slope of $-(1/K_D)$. Note, the x-intercept is equal to B_{MAX}. Figure 5 illustrates these concepts. The advantage of the Scatchard plot is that if the binding interaction does in fact satisfy all the assumptions for a single class of binding sites, the linearized graph is relatively easy to analyze for the binding parameters. However, if the Scatchard plot is not linear, as is often the case, this approach is not the best for estimating numerical values for binding parameters.

If more than one class of binding sites are present, then Equation 12 applies (for the case of two classes of binding sites) and the Scatchard transformation becomes

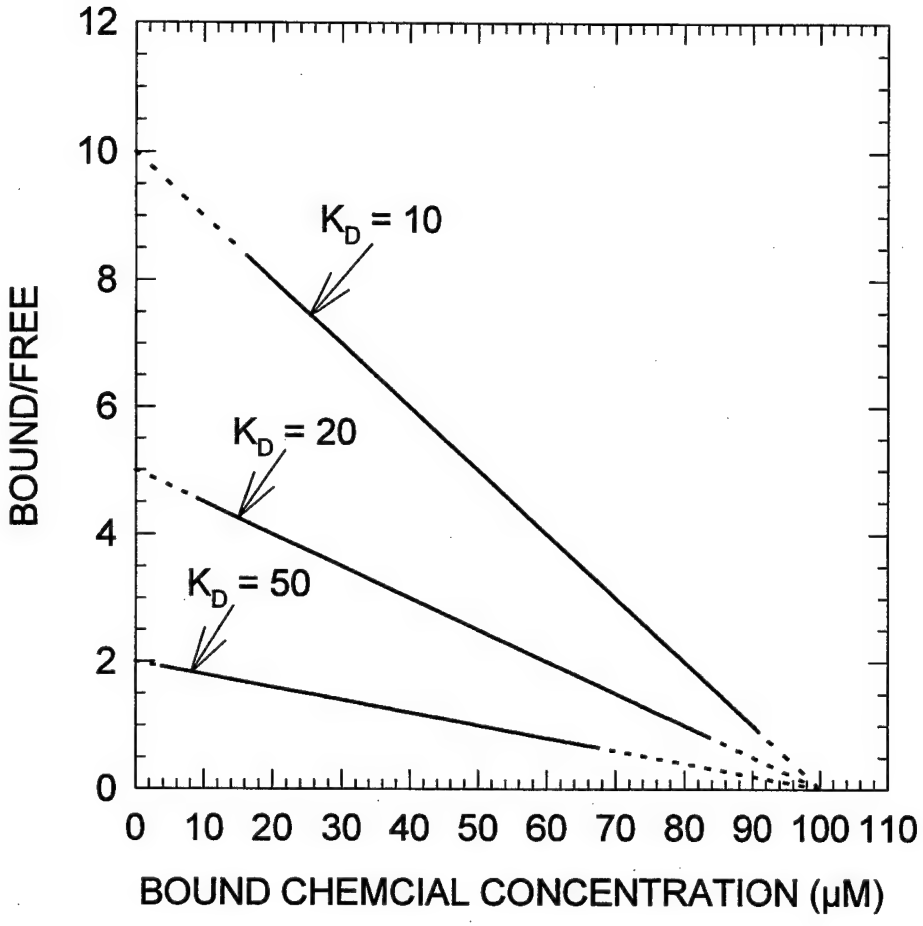

Figure 5: Standard Scatchard plot - single class of binding sites. The Scatchard plots for three cases are shown, B_{MAX} = 100μM and K_D = 10, 20 and 50 μM.

$$\frac{B}{F} = \frac{1}{2} * \left(\alpha - \beta * B + \sqrt{(\alpha - \beta * B)^2 + 4 * \delta * B} \right) \quad (19a)$$

where

$$\alpha = \frac{B_{MAX1}}{K_{D1}} + \frac{B_{MAX2}}{K_{D2}} \quad (19b)$$

$$\beta = \frac{1}{K_{D1}} + \frac{1}{K_{D2}} \quad (19c)$$

and

$$\delta = \frac{B_{MAX1} + B_{MAX2}}{K_{D1} * K_{D2}} \quad (19d)$$

This relationship is much more complicated than the case of one class of binding sites. The plot of Equation 19a (Figure 6) is curvilinear and the non-linearities lead to significant difficulties in analyzing the graphical data to evaluate the binding parameters, particularly if there are experimental errors. One advantage of the Scatchard plot, as illustrated by Figure 6, is that it provides a sensitive visual method to identify the presence of multiple binding sites.

Figure 6 illustrates the expected behavior for the Scatchard plot when two binding sites are present. Inspection of Equation 19a indicates that the intercept with the y-axis (set B = 0) is α, i.e., the sum of the ratios of the capacity of each binding site to the affinity (Equation 19b). The x-intercept must be the maximum value for the total binding which is the sum of the capacities of all the binding sites, $B_{MAX1} + B_{MAX2}$. If there are more than two classes of binding sites, then the analysis becomes more complicated and it is usually very difficult to identify the various classes of binding sites and to uniquely determine the binding parameters.

One experimental issue relates to how the concentration of B is expressed. In the analysis above, it is assumed that the endogenous molecule containing the binding site is in solution and when the chemical is bound to the binding site the complex remains in solution. Thus, B is expressed in μM and has the normal interpretation of a concentration of a solute in solution, where the solute is the chemical-binding site complex. If the binding study is to evaluate the interaction of the chemical with a particular endogenous molecule, such as a plasma protein (e.g., albumin), and the conditions of the reactions are under the control of the investigator such that the concentration of the endogenous binding molecule is known, then the usual procedure is to divide both sides of Equation 18 by the total concentration of the endogenous

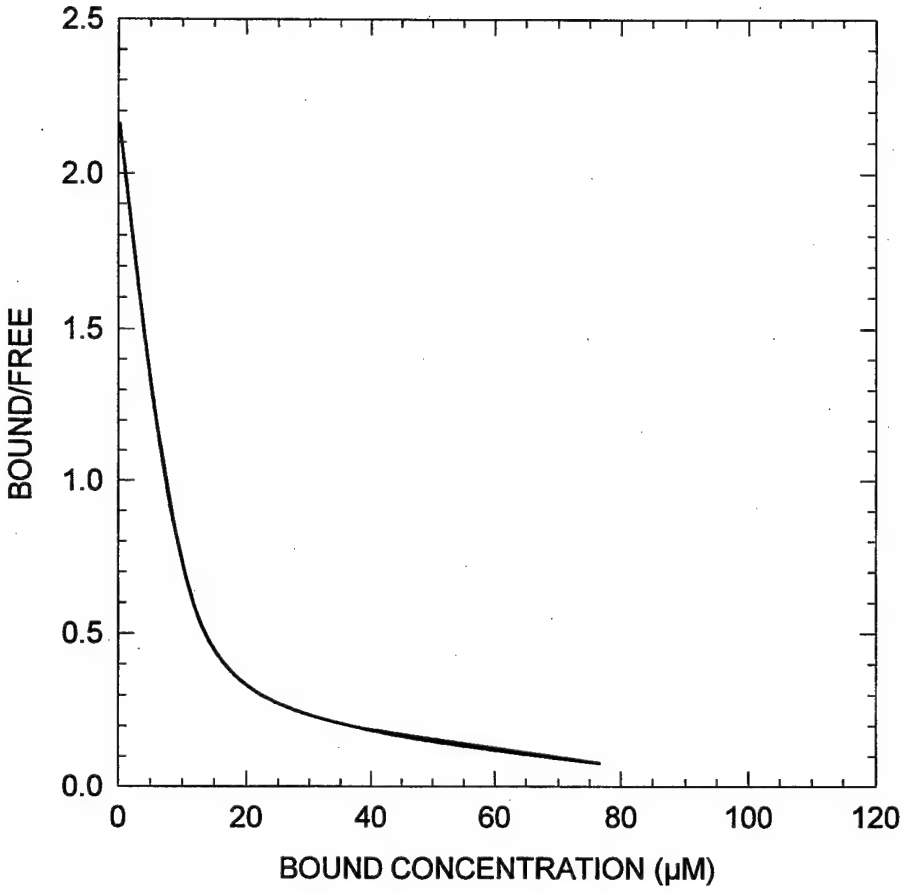

Figure 6: Standard Scatchard plot - two classes of binding sites. The case illustrated is for two classes of binding sites, one high affinity low capacity and the other low affinity high capacity. Specifically the first class of binding sites has B_{MAX1} = 10 μM and K_{D1} = 5 μM and the second class of binding sites has B_{MAX2} = 100 μM and K_{D2} = 500 μM. The y-intercept should be 2.2 and the x-intercept should be 110 μM.

binding molecule in the reaction mixture, L_0 {μM}. If we define R {dimensionless} as the ratio of B to L_0, then R is the average number of molecules of the exogenous chemical bound to each endogenous molecule at equilibrium and B_{MAX}/L_0 is the maximum number of binding sites on each endogenous molecule, denoted n. Thus, Equation 18 becomes

$$\frac{R}{F} = \frac{n}{K_D} - \left(\frac{1}{K_D}\right) * R \qquad (20)$$

Expressing the bound concentration in these units results in the y-axis having units of μM^{-1} and the x-axis being dimensionless. Note, the y-intercept is now n/K_D and the x-intercept is n, the number of (identical) binding sites per endogenous binding molecule. This analysis can only be performed when the concentration of the endogenous molecule is known in units of molarity.

An additional complication with units occurs when the binding study involves complex mixtures of potential binding molecules, such as plasma or tissue homogenates. In this case, the actual concentration of the endogenous binding molecule is not usually known. In fact, the identity of the molecule may be unknown. Hence, L_0 is undefined. If it is assumed that the molecule is soluble, then the Scatchard analysis using B and F can be applied and B_{MAX} and K_D determined. In this situation, B_{MAX} represents the total concentration of binding sites without distinguishing whether there are multiple sites on the endogenous binding molecule. The likelihood that the complex mixtures of biological materials will behave as a single class of molecules is small. Also, if the endogenous binding molecule is not soluble, which may be the case in tissue homogenates, the analysis will be confounded. See the discussion in Section 3.1.4 (Experimental Complications) for methods to investigate this issue.

Under certain conditions, the B_{MAX} must be extrapolated back to the *in vivo* situation to have meaning. No extrapolation is needed if undiluted biological fluids, e.g., plasma, is used. However, if plasma is diluted or tissue homogenates are used, then an extrapolation to zero dilution is required. See Section 6.1 - Appendix A for details.

The value of the Scatchard plot is to improve the likelihood of identifying the presence of multiple classes of molecules by visual inspection. If the range of concentrations used in the experimental studies includes concentrations high enough to attain saturation of all binding sites, then the Scatchard plot will clearly test the hypothesis that a single class of binding sites is present, i.e., the plot will be a straight line. If the Scatchard plot is linear but the range of concentrations tested does not elicit the saturation situation, then there is some question as to whether there may be additional classes of binding sites that are not resolved at the lower concentrations tested. If the Scatchard plot is obviously curvilinear, then the possibility of multiple classes of binding sites and/or interactions between binding sites must be investigated.

While Scatchard plots are very useful for visualizing data in general, they are not the most accurate way to analyze data if more than one class of molecules are present.

The problem is that the transformation of the data distorts the experimental error. Linear regression analysis assumes that the distribution of data points around the true line is Gaussian and that the standard deviation is the same at all x-values. These assumptions are not true with the transformed data. In addition, the Scatchard transformation results in the x and y values being interdependent; the y-value, B/F, is dependent on the x-value, B. Since the assumptions of linear regression analysis are violated, the binding parameters, B_{MAX} and K_D, determined by linear regression of the Scatchard transformed data are more biased than if the parameters are determined by non-linear regression of the untransformed data. Considering the time and effort put into collecting binding data, the best possible analysis technique should be employed. Non-linear regression analysis of B versus F produces the most reliable results. Scatchard analysis produces approximate estimates of binding parameters and can be used effectively as starting values for the non-linear fitting routines.

In general, a two binding site model will fit the experimental data better than a one binding site model. Mathematically, the additional parameters provide more degrees of freedom which allows for a better fit, i.e., reduction in the total error. The question arises as to whether the reduction in total error resulting from going to the more complex model has any meaning. If a goodness of fit analysis for the one binding site model shows that the data are consistent with the model, i.e., if a Chi-squared analysis indicates that the experimental data do not deviate from the predicted values of the model by more than what could be expected by chance (see Press, et al., 1989 - Chapter 14), then even though the two binding site model reduces the total error, the improvement does not justify the added complexity of the two binding site model. Additional studies would be required to "prove" that there exists an additional class of binding sites.

Furthermore, when considering selection of a two (or more) binding site model over a one binding site model, it is important to evaluate whether the higher order model has biological significance as opposed to mathematical significance. It is recommended to disregard a two-binding site model fit when:

(i) the two K_D values are almost identical, or
(ii) one of the K_D values is outside the range of the experimental data.

In the second case, the possibility of non-specific binding should be explored. If the two (multi) binding site model seems to be a more reasonable fit to the data, then a test of whether the improvement in fit is statistically significant is necessary (as discussed above).

3.1.4 Experimental Complications in Titration Experiments

Adsorption of chemical to surfaces

Many chemicals and molecules will bind to plastic, metal or glass surfaces. The adsorption process depends on the physical-chemical nature of the surface and the

chemical/molecule of interest. Binding of either the chemical and/or the endogenous molecule of interest to the surfaces of the experimental apparatus can lead to errors in the calculation of binding parameters. Before any binding studies are conducted, the compatibility of the chemical and binding molecule with the experimental system should be investigated. A simple control experiment with chemical present but no binding molecule will indicate whether the chemical will bind to the apparatus. If binding of the chemical to a purified binding molecule is to be studied, then a pure solution of the molecule without the chemical can be run as a control and any loss of binding molecule can be determined. However, if an unknown mixture of binding molecules, such as tissue homogenates, is under investigation, then the question of the stability of the endogenous molecule is difficult to evaluate. In this case, some surrogate measure of binding molecule concentration, such as total protein concentration, should be evaluated as a general indicator for binding molecule stability.

If there is no loss of chemical or binding molecule in control experiments, then the binding studies can proceed. However, if losses are observed then care must be taken to assure that this effect will not influence the estimation of binding parameters. If the losses are small and the concentrations of free chemical, total chemical and total binding molecule are determined by analytical methods in each reaction, then the results of the analysis should be correct. If the adsorption losses are significant and/or time dependent, then it may be necessary to obtain reaction vessels constructed of materials that do not bind chemical and/or binding molecule or, alternatively, block adsorption by chemical treatment of the apparatus.

Non-specific binding to binding molecule

From the practical point of view of how chemical binding affects the kinetics of chemicals in biological systems, the distinction between specific and non-specific binding is not particularly important. If the chemical is bound to a biomolecule, whether specifically or non-specifically, it is not available to participate in other kinetic processes such as transport or metabolism. However, if concern about the interactions of chemical in mixtures is important, then the possibility of one chemical displacing another chemical becomes a relevant issue. In this case, non-specific binding is defined as the proportion of chemical binding to an endogenous molecules that is not displaced by other structurally similar chemicals. Thus, when performing binding experiments where an understanding of the nature of specific binding is of interest to allow for quantitative predictions of chemical interactions, it will be necessary to measure both total binding and non-specific binding, and calculate specific binding as the difference.

One technique for evaluating non-specific binding involves using competitive binding inhibitors. For each concentration of the chemical, two reaction vials are prepared as described above for titration studies. One reaction is used to measure total binding. The second reaction is conducted in the presence of an excess of a competitor for the specific binding site that competitively saturates the specific binding site (i.e., displaces the chemical of interest from the specific binding site), thus bound chemical remaining is a measure of non-specific binding. The concentration of the competitor

used should be great enough to block virtually all specific binding of the chemical of interest, but not so much that physical changes to the endogenous molecule may occur. A useful rule of thumb is to use the competitor at a concentration equal to 100 times its K_I for the specific molecule. The corrected data, total bound concentration (from the first reaction vessel) minus the non-specific bound concentration (from the second reaction vessel), can be used to evaluate the binding parameters for the specific binding site.

Endogenous inhibitors

In some binding studies, particularly those that involve biological materials such as plasma and tissue homogenates, it is possible that there may exist endogenous chemicals that will bind to the same binding molecule as the chemical of interest. In this case, there is the possibility that the observed K_D will not be the true K_D but in fact an apparent K_D. If the concentration of the endogenous inhibitor is constant under all physiologically relevant situations, then the apparent K_D as determined by the standard titration methods described above, when extrapolated back to the zero dilution situation, is the correct parameter to use in the BBK model, i.e., the apparent equilibrium dissociation constant takes into account the effect of the endogenous inhibitor on binding of the chemical of interest to the endogenous binding molecule. If on the other hand, the endogenous inhibitor can vary under physiological conditions, then it may be necessary to evaluate the true binding parameters for both the chemical and the inhibitor and solve the competitive inhibition relationships (Equation 16a and 16b) to predict the free concentration of the chemical of interest in various compartments of the model. In this latter case, it will be necessary to know the total concentration of the endogenous inhibitor in each scenario modeled.

The presence of a competitive inhibitor can be readily detected by carrying out the binding study using a series of dilutions of the biological component. If no endogenous inhibitors are present, the binding curves, when normalized by B_{MAX}, should be identical for all dilutions. This can be explained by inspecting Equation 6a. In the absence of an inhibitor, the effect of dilution is to reduce B_{MAX} and thus reduce the amount of chemical bound. But the ratio of B to B_{MAX} for a given free concentration is independent of dilution. On the other hand, if an endogenous inhibitor is present then Equation 15a applies. In this case, the effect of dilution is not only to reduce B_{MAX} but will also reduce the concentration of the inhibitor, F_I. Now the ratio of B to B_{MAX} at a given free concentration will depend on dilution since F_I changes with dilution. If normalized binding is observed to increase with dilution, then the presence of an endogenous inhibitor should be considered.

Metabolism

If the chemical of interest is unstable or is metabolized by enzymes present in the tissue whose binding properties are under investigation, then experimental complications may arise due to the disappearance of the chemical over time. The stability of the chemical should be investigated by independent control experiments prior to conducting the binding studies. If the rate of metabolism is zero or very slow relative

to the time required to reach equilibrium in the binding experiment, then this will not be an issue. However, if the rate of metabolism is comparable or faster than the time constant for binding equilibration, then the effect of metabolism must be taken into consideration.

In this situation, the ultrafiltration method for binding is preferable since diffusion is not a limiting factor. Furthermore, if possible, metabolism should be blocked either by a metabolic inhibitor or physical treatment, e.g., kinetic studies at 4°C. However, this approach will always introduce confounding factors: does the metabolic inhibitor compete for binding, can the metabolic inhibitor bind the chemical of interest, does physical treatment affect the endogenous binding molecule, etc.? There are no simple solutions to this problem and each case must be investigated individually.

Non-soluble molecules

When evaluating the binding of a chemical to molecules present in tissue homogenates, the possibility arises that the chemical binds to a molecule that is not in the solution phase, i.e., is associated with the membrane phase. The problem is not so much in conducting the binding experiments, although the presence of the solid phase in tissue homogenates can create technical problems, e.g., clogging of ultrafiltration membranes, but in the interpretation of the binding in the context of the *in vivo* situation. One way to determine if binding to the membrane phase is important is to conduct parallel binding studies using both whole tissue homogenate and cytosol (supernatant separated from homogenate by high speed centrifugation). If binding only occurs in whole homogenate and not in cytosol, then it is clear that the endogenous binding molecule is present in the solid phase. If, on the other hand, binding occurs in both samples and the amount of binding in the cytosol can account for the binding in the whole homogenate, then it can be concluded that the binding molecule is located in the soluble phase of the cell. More likely, there will be a distribution of binding between the phases.

Assuming that in the preparation of tissue homogenates for binding studies, the tissue vasculature was perfused to remove blood from the tissue (i.e., the tissue homogenate was not contaminated with plasma or RBCs), then the binding in the soluble phase can be interpreted in terms of the BBK model as binding in the interstitial or intracellular water compartment. Binding to the solid phase is more difficult to interpret. Plasma membrane associated molecules could be either exo- or endo-oriented. If they are on the cell surface, then binding will influence the free concentration of the chemical in the interstitial water compartment. If the binding site is associated with the inside surface of the plasma membrane, i.e., endo-oriented, and/or associated with organelle membranes, then the relevant chemical concentration impacted would be in the intracellular water space. Additional studies are required to differentiate these two possibilities.

Ionic strength of buffers

The ionic strength of the solution in which the chemical and the binding molecule exist can have a significant impact on the strength of the binding interaction as represented by K_D. This effect will be particularly important if ionic binding is involved. The strength of an ionic bond between two charged groups will be affected by the ionic strength of the solution in which they exist. Therefore, the ionic strength of the buffers used in the binding study should approximate that of the relevant *in vivo* compartment in order to give physiologically relevant binding parameters.

pH Effects

pH can have an effect on binding of a chemical to a biological molecule. If the charge of the chemical and/or the binding molecule is affected by pH, then this factor can have profound effects on the binding interaction. As with the ionic strength effect, the experimental resolution is to conduct the binding experiments at the physiologically relevant pH.

Temperature Effects

Temperature can influence the observed binding parameters through several routes. In general, the binding capacity will not be affected by temperature, unless the solubility of the binding molecule becomes an issue. However, the equilibrium dissociation constant can be affected. Since K_D is the ratio of two micro-rate constants, each of which could be temperature dependent, then if the temperature coefficients for the two micro-constants are different, the K_D will change with temperature. The best situation would be to conduct the binding experiment at physiologically relevant temperatures, i.e., 37°C. If this is not possible, then the experiments should be conducted at two different temperatures and the effect of temperature evaluated empirically.

3.2 INHIBITION EXPERIMENTS

3.2.1 Experimental Design of Inhibition Experiments

The objective of inhibition studies is to develop an understanding of the nature of the molecular binding site. By determining what structurally related chemicals will compete for binding to the endogenous molecule, the structural determinants of the binding site can be resolved. Thus, the requirements for charge groups, polarizable groups, hydrophobic regions and their relative physical dimensions can be determined. Binding of a chemical by the endogenous molecule is not sufficient evidence alone to assume that it will compete with other chemicals for binding to the same binding molecule. Large macromolecular molecules may have more than one class of independent binding sites that can interact with high specificity with different chemicals without inhibitory interactions between the chemicals. However, if two chemicals withsome degree of chemical similarity bind to the same endogenous molecule, it would be reasonable to explore the possibility of competitive binding inhibition.

The usual competitive binding inhibition experiments are conducted with the concentration of the molecule and the inhibitor held constant and the concentration of the chemical of interest varied over a range of concentrations, i.e., the standard titration experiment in the presence of a fixed concentration of the inhibitor.

3.2.2 Data Analysis for Inhibition Experiments

The binding curve for each inhibitor concentration should be plotted using a Lineweaver-Burke transformation, a 1/B versus 1/F plot. Analysis of this plot is based on the relationship

$$\frac{1}{B} = \frac{1}{B_{MAX}} + \left(\frac{K_D\left(1 + \frac{F_I}{K_I}\right)}{B_{MAX}} \right) * \frac{1}{F} \qquad (21)$$

which is derived from Equation 15a. Note, if the concentration of the inhibitor is zero, $F_I = 0$, then this equation reduces to a form that is equivalent to the standard Lineweaver-Burke equation for enzyme kinetics. This plot is the quickest way to get a visual idea of the nature of the binding inhibition (Figure 7). If the inhibition appears to be competitive, then fitting of the individual binding curves by the non-linear regression analysis will provide estimates of the apparent K_D for each of the inhibitor concentrations used. If a plot of the apparent K_D versus the free concentration of the inhibitor is constructed, the y-intercept should equal the true K_D and the slope should equal K_D/K_I.

One complication in conducting this analysis is the fact that it is the free concentration of the inhibitor that is needed for the plot. Several possible solutions for this problem exist. If the free concentration of the inhibitor is measured in the binding experiment, then this quantity is known directly. Another approach is to determine the binding parameters for the interaction of the inhibitor with the molecule, i.e., conduct an independent binding study of the inhibitor. Using the measured binding parameters for both the chemical and the inhibitor, the free concentration can be calculated and used in the apparent K_D versus free inhibitor concentration plot. Either one of these approaches should work. Then, the plot of the predicted "apparent" K_D versus the observed "apparent" K_D can be used to confirm the competitive nature of the interaction.

If the interaction between the inhibitor and the chemical of interest for binding is not competitive, then additional analysis will be necessary.

3.3 KINETIC EXPERIMENTS

The purpose of kinetic studies is to evaluate the forward and reverse rate constants, k_+ and k_- independently. If the actual rates of the forward and reverse reactions, as controlled by the rate constants and concentrations of reacting species, is rapid (rates more than 10 fold faster than other relevant processes, e.g., metabolism

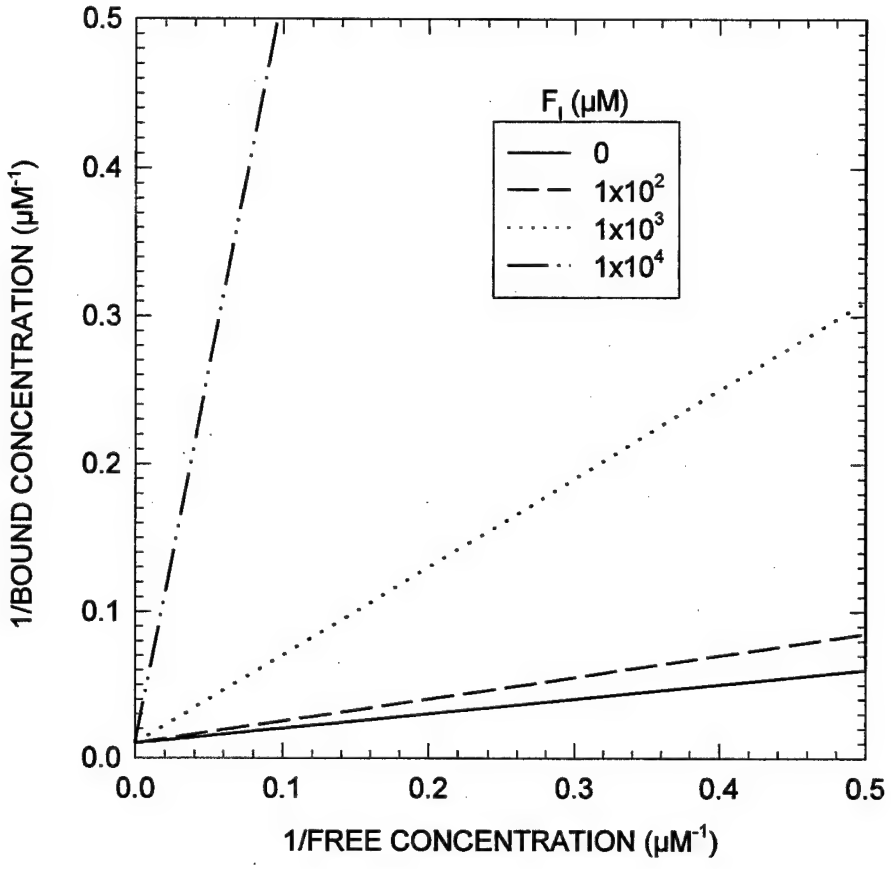

Figure 7: The effect of an inhibitor on the Lineweaver-Burke plot - single class of binding sites. The chemical of interest has binding parameters of B_{MAX} = 100 μM and K_D = 10 μM; the inhibitor has binding parameters of B_{MAXI} = 100 μM and K_{DI} = 200 μM. The free concentration of the inhibitor varies from $1x10^1$ to $1x10^4$ μM.

and membrane transport), then assuming that binding is in equilibrium will have minimum impact on kinetic predictions. However, if the rates are comparable or slower, then binding reactions may be rate limiting and have a significant impact on kinetic behavior. Although these issues are important, the experimental basis for evaluating micro-rate constants will not be discussed here.

4.0 EXAMPLE - TCA BINDING TO BOVINE SERUM ALBUMIN

In order to illustrate some of the issues discussed above, the binding of trichloroacetic acid (TCA) to bovine serum albumin (BSA) in a Krebs-Ringer bicarbonate buffered solution (isolated perfused rat liver (IPRL) perfusion medium) will be discussed. In all of the studies presented, the nominal concentration of albumin is 40 g/L (4 percent weight to volume). Using 69K as the molecular weight of BSA, this corresponds to a nominal BSA concentration of 580 μM. The binding studies were conducted as discussed above using ultrafiltration as the technique to separate free chemical from bound chemical.

Figure 8 presents the standard binding curves for TCA binding to BSA obtained in three different experiments. The data indicate that the binding relationship is reproducible. The maximum concentration of TCA that can be used without significant precipitation is approximately 16 mM. Up to that concentration saturation is not observed. In order to confirm that there are no competitive inhibitors present in the perfusion medium, binding was evaluated at different dilutions of the perfusion medium. The results are shown in Figure 9. At four different protein concentrations over a 20-fold range the binding curve is relatively constant. The lower protein concentrations result in more error since the bound concentration is determined by the difference between the total concentration and the free concentration. At low protein concentrations and high TCA concentrations, most of the chemical is free and the calculation of bound TCA is the result of the difference of two numbers that are almost identical. Thus, small errors in the free and total concentrations lead to large errors in the calculated bound concentration.

In order to investigate whether the binding could be described by a single class of binding sites, the Scatchard plot was constructed (Figure 10). It is obvious that more than one class of binding sites are present. In this case, as was noted in the discussion of multiple classes of binding sites, the fitting of the binding data should be conducted using a non-linear fitting routine with the un-transformed data. Figure 11 illustrates the results of investigating two different models to fit the binding data. One model is a combination of a single specific binding site and a linear non-specific binding site (B_{MAX} = 1734 μM, K_D = 396 μM, α = 0.224). The other model was with two different specific binding sites and a linear non-specific binding site (B_{MAX1}= 217 μM, K_{D1} = 9.2 μM, B_{MAX2} = 2576 μM, K_{D2} = 1337 μM, α = 0.154). The latter case gives the best fit.

Finally, using the parameters for the best fit, the relationship between the free concentration and the total concentration can be determined. Figure 12 shows this

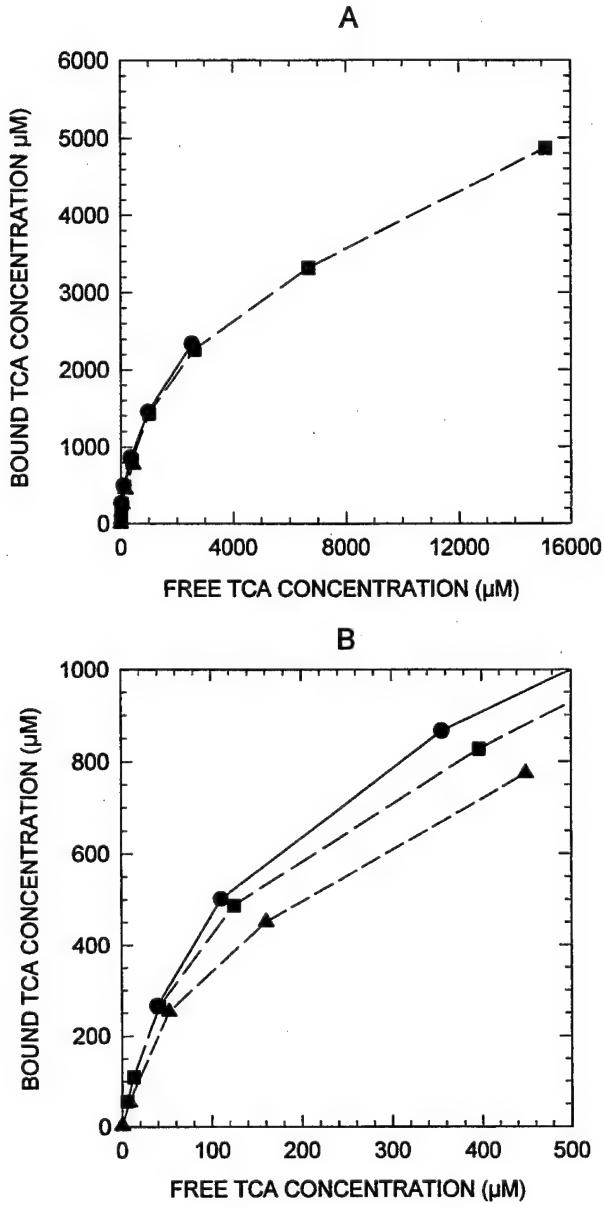

Figure 8: Binding of TCA to BSA in isolated perfused rat liver perfusion medium. Binding studies were conducted as described in the text. BSA concentration in the perfusion medium was 580 µM. (A) Standard binding curve over full range of concentrations used in binding study. (B) Expanded view of binding curve in the low concentration range. Three separate binding studies were conducted.

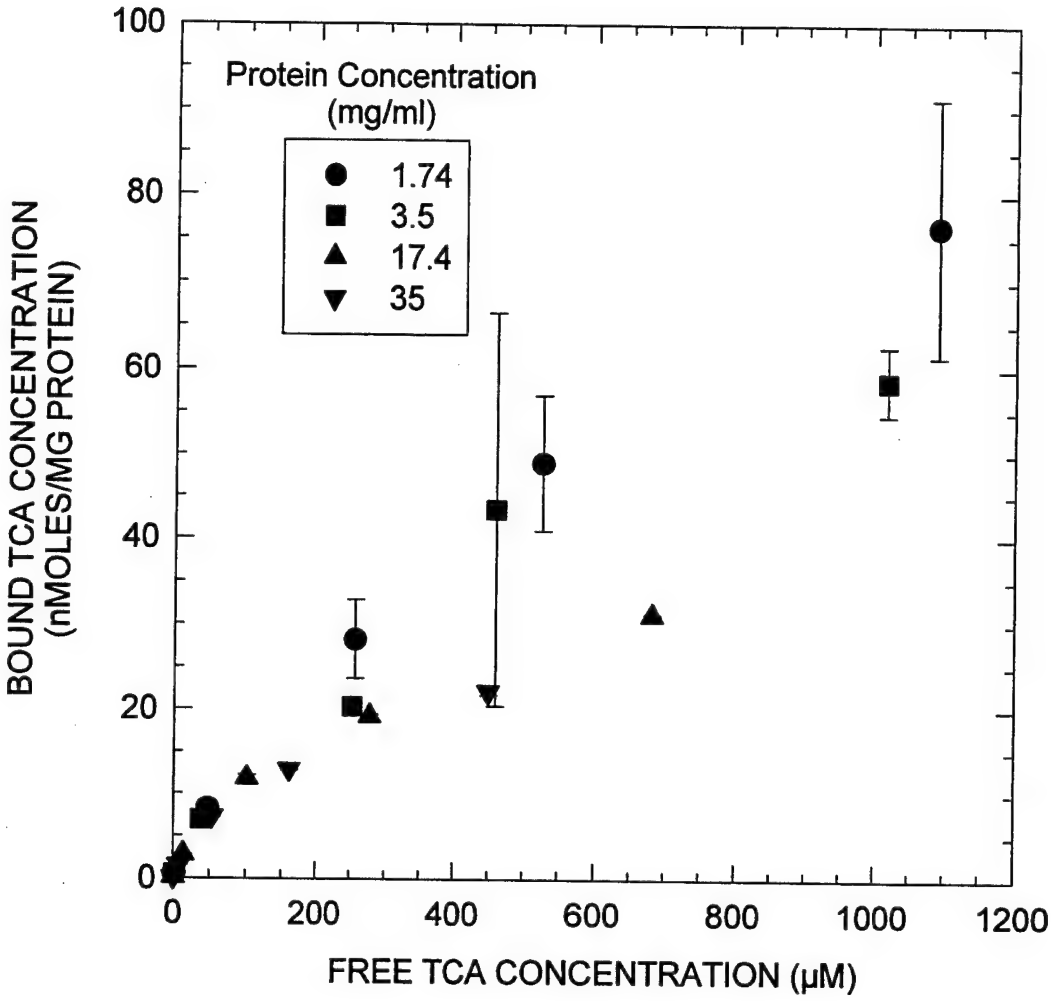

Figure 9: Effect of dilution on binding of TCA in isolated perfused rat liver perfusion medium. Binding studies were conducted at four different protein concentrations. Binding data were normalized per mg protein.

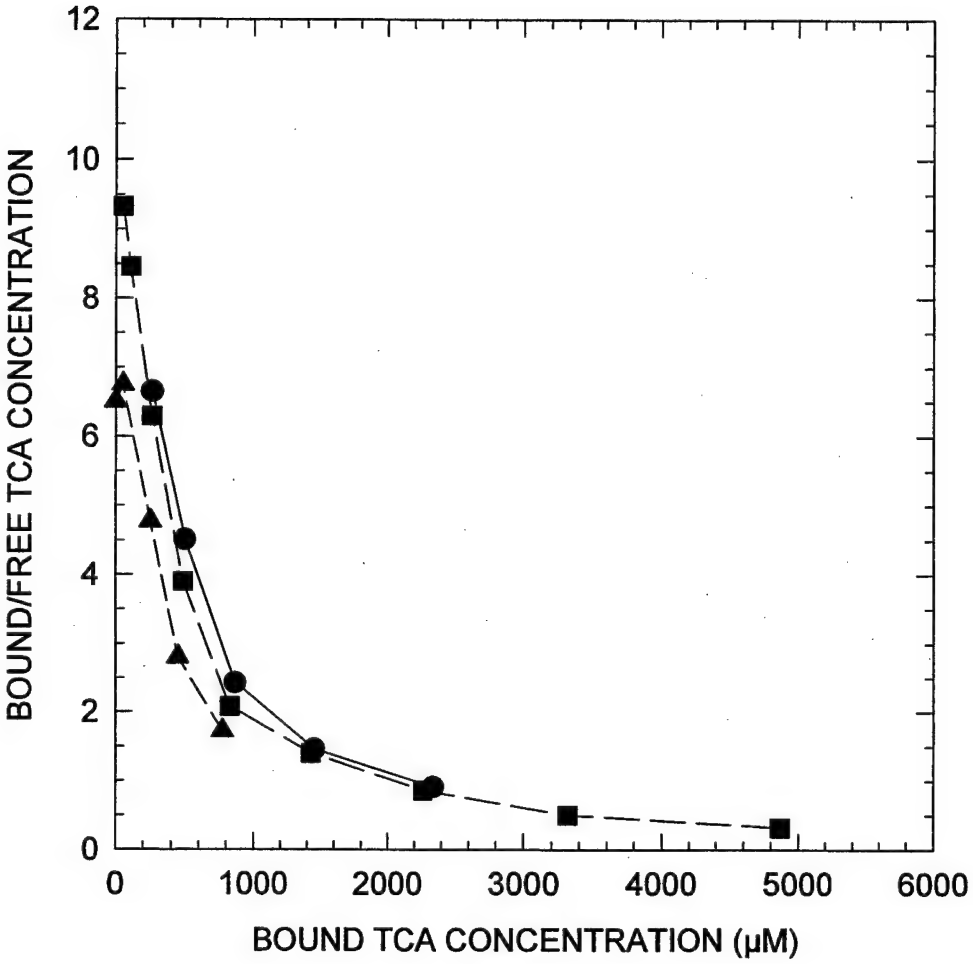

Figure 10: Scatchard plot for TCA binding in isolated perfused rat liver perfusion medium. Data are the same as that used in Figure 8.

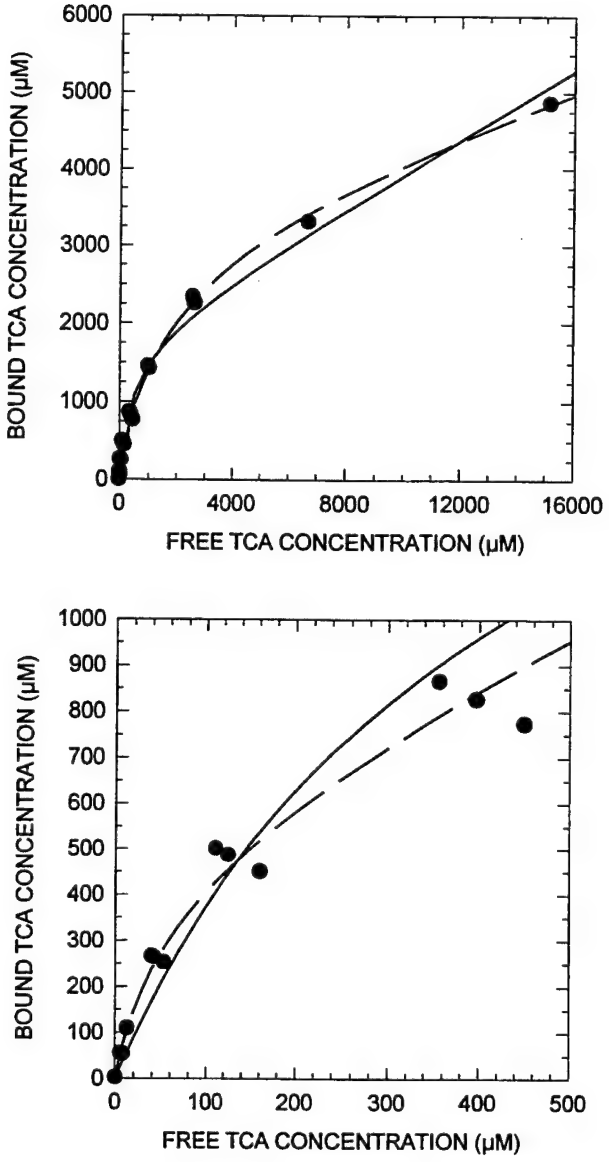

Figure 11: Fitting of two binding models to the TCA binding data. (A) Standard binding curves for the full range of concentrations used. (B) Expanded view of the binding curve in the low concentration range. Solid lines indicate predictions for the single binding site plus non-specific binding model (B_{MAX} = 1734 μM, K_D = 396 μM, α = 0.224); dashed lines indicate predictions for the two binding site plus non-specific binding model (B_{MAX1}= 217 μM, K_{D1} = 9.2 μM, B_{MAX2} = 2576 μM, K_{D2} = 1337 μM, α = 0.154).

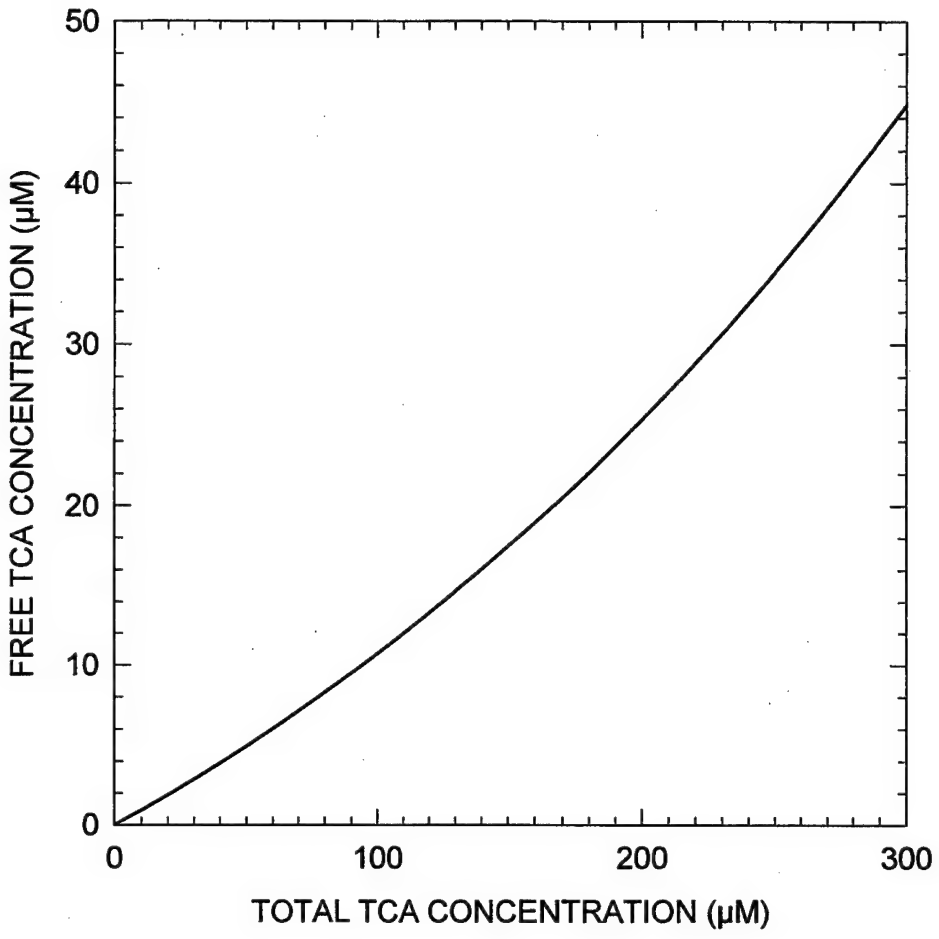

Figure 12: Free concentration of TCA versus total TCA concentration in the IPRL perfusion medium using the two binding sites plus non-specific binding model.

relationship where the predicted free TCA concentration is plotted as a function of the total TCA concentration for the perfusion medium used in the IPRL studies. Binding of TCA to BSA suppresses the free concentration to less than 15% of the total concentration. This effect will have a significant impact on TCA kinetics.

5.0 REFERENCES

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6.0 APPENDICIES

6.1 Appendix A: Calculation of concentration of binding sites in tissue water spaces

The location of the endogenous binding molecules in a given tissue is an issue of concern. The biomolecule can be located in any one of the tissue water spaces (the vascular space, the interstitial space or the intracellular space) or the solid phase of the tissue (cellular membranes or connective tissue). In this discussion it is assumed that the binding molecule is soluble, i.e., not bound to the solid phase.

If the chemical is bound to plasma proteins, then the binding parameters for the vascular space can be obtained from binding studies using plasma. The parameters from these studies can be used in BBK models for the vascular space of the tissue. Furthermore, if there is binding to plasma proteins, then it can be assumed that binding will occur in the interstitial space in proportion to the concentration of plasma proteins in the interstitial fluid. Additional binding molecules may exist in the interstitial space due to secretion from the parenchymal cells of the tissue. The question of protein binding in the vascular space can be eliminated by perfusion of the tissue with a buffer solution prior to homogenizing the tissue for the binding studies. If there is known binding of the chemical to plasma proteins, then the effect of binding to plasma proteins in the interstitial space must be taken into consideration. Finally, binding to intracellular proteins using tissue homogenates can be directly investigated.

Assuming the binding molecule is a soluble protein in the parenchymal cell cytosol of the ith tissue and the concentration of the binding site in that water space is B_{MAXi}, then the total number of binding sites in a sample of tissue of weight W_i {g} is

$$N_S = B_{MAXil} * V_{il} = B_{MAXil} * FV_{il} * W_i \qquad (A1)$$

where FV_{il} {mL/g} is the conversion factor for volume of interstitial water per weight of tissue. This tissue sample is homogenized in a volume of buffer determined by the weight of the tissue sample,

$$V_B = d_1 * W_i \qquad (A2)$$

where d_1 {mL/g} is the volume of buffer per weight of tissue (3-4 mL/g). Thus, the concentration of the binding sites in the water phase of the homogenate is

$$B^* = \frac{B_{MAXil} * FV_{il} * W_i}{\alpha * W_i + d_1 * W_i} = \frac{B_{MAXil} * FV_{il}}{\alpha + d_1} \qquad (A3)$$

where α {mL/g} is the total volume of tissue water per weight of tissue including vascular, interstitial and intracellular water (approximately 0.8 mL/g for soft tissues). Since succeeding steps utilize volumetric samples of homogenate that contain both

water and solid phases, the fractional water volume of the homogenate (the water volume per unit volume of original homogenate) is needed. This is given by

$$\beta = \frac{\text{water volume}}{\text{total volume}} = \frac{\alpha * W_i + d_1 * W_i}{\frac{W_i}{\rho} + d_1 * W_i} = \frac{\alpha + d_1}{\frac{1}{\rho} + d_1} \qquad \text{(A4)}$$

where ρ {g/mL} is the density of the tissue.

In most cases, the homogenate is diluted further before the binding studies are conducted. The concentration of the binding sites in the water phase of the diluted homogenate is

$$B^{**} = \frac{B^* * \beta * V_H}{\beta * V_H + d_2 * V_H} = \frac{B_{MAXil} * FV_{il}}{(\beta + d_2) * \left(\frac{1}{\rho} + d_1\right)} \qquad \text{(A5)}$$

where Equation A3 was substituted for the concentration of binding sites in the water phase of the homogenate and Equation A4 was used to eliminate β in the numerator, V_H {mL} is the volume of the aliquot of original homogenate diluted and d_2 {dimensionless} is the volume of dilution buffer added per volume of original homogenate diluted. The water volume fraction will again be needed below and is given by

$$\gamma = \frac{\beta * V_H + d_2 * V_H}{V_H + d_2 * V_H} = \frac{\beta + d_2}{1 + d_2} \qquad \text{(A6)}$$

The next step is to set up the binding reaction. A given volume, V_R, of the diluted homogenate is placed in a reaction vessel and a volume, V_S, of stock solution of the chemical of interest is added. The concentration of the binding sites in the final reaction mixture is given by

$$B^{***} = \frac{B^{**} * \gamma * V_R}{\gamma * V_R + V_S} = \frac{B_{MAXil} * FV_{il}}{\left(\frac{1}{\rho} + d_1\right) * (1 + d_2) * (\gamma + d_3)} \qquad \text{(A7)}$$

where d_3 {dimensionless) is the ratio of V_S to V_R. The reaction is allowed to proceed for a given period of time sufficient to allow equilibrium to be attained. The total and free concentration of the chemical are determined by analytical techniques and the binding data are analyzed by the techniques discussed above. The experimental determined concentration of binding sites should be equal to the concentration of binding sites defined by Equation A7. Solving for B_{MAXil} will provide an estimate of the concentration of binding sites in the intracellular space of the tissue. Thus,

$$B_{MAXil} = \left(\frac{1}{FV_{il}}\right) * \left(\frac{1}{\rho} + d_1\right) * (1 + d_2) * (\gamma + d_3) * B_{MAX}^{EXPT} \quad \text{(A8)}$$

One technique to confirm that the binding sites are in the soluble phase is to conduct the binding study with tissue cytosol, i.e., the water phase obtained by centrifuging the original tissue homogenate. If this is done, the concentration of the binding sites in the original preparation of the cytosol is given by Equation A3. The cytosol may be used directly or diluted again introducing a factor $(1 + d_2)$ where d_2 {dimensionless} is the volume of diluent buffer per volume of cytosol. When the binding reactions are set up, the cytosol is again diluted and the effect is accounted for by a factor of $(1 + d_3)$ Thus, the estimate for the concentration of binding sites in the intracellular space of the tissue using cytosol is

$$B_{MAXil} = \left(\frac{1}{FV_{il}}\right) * (\alpha + d_1) * (1 + d_2) * (1 + d_3) * B_{MAX}^{EXPT} \quad \text{(A9)}$$

The differences in the formulas for calculating the binding capacity in the intracelular space of the tissue using homogenate or cytosol relate to the presence of the solid phase in the tissue homogenates. However, if the binding is in the soluble phase, then the two estimates for binding capacity should be equal, within experimental errors. If the estimate for binding capacity using the tissue homogenate is significantly greater than the estimate using the cytosol, then the possibility of significant binding to the solid phase, membranes and/or connective tissue, must be considered.